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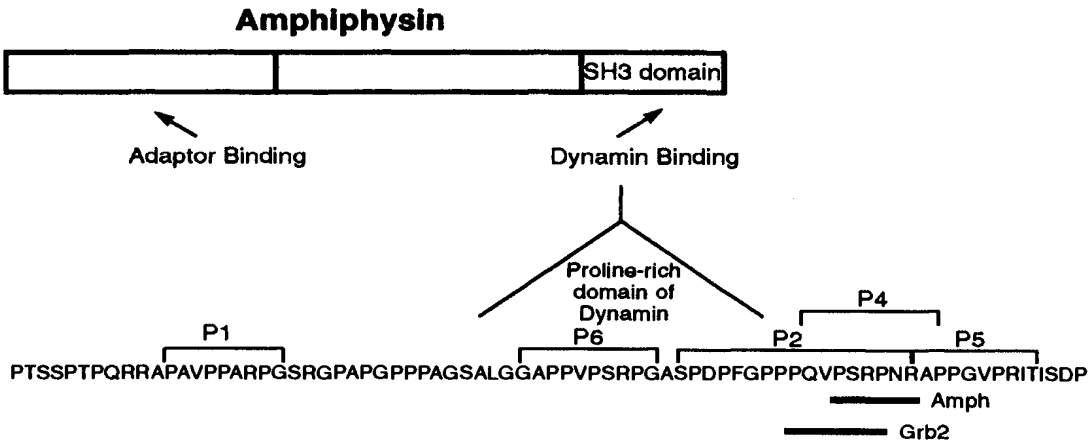

(57) Abstract

Disclosed is a membrane permeable molecule which inhibits dynamin–mediated endocytosis in an eukaryotic cell, the molecule comprising a membrane permeable hydrophobic moiety, and a peptide moiety comprising the amino acid sequence QVPSRPNRAP.

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Title: Improvements in or Relating to Uptake of Substances by Cells

Field of the Invention

This invention relates to a novel molecule and to various uses thereof.

Background of the Invention

Eukaryotic cells secrete substances from membrane-bound vesicles present in the cytoplasm by migration of the vesicles to the cell membrane. Fusion of the vesicle membrane with the cell membrane allows the vesicle contents to escape into the extracellular environment. This process is termed exocytosis.

The process by which cells can take up certain substances involves a different mechanism and is termed endocytosis.

Exocytosis and endocytosis are performed by many different cell types and are involved in many physiological processes (e.g. secretion of digestive enzymes, secretion of neurotransmitters and hormones, cell surface receptor downregulation/internalisation, retrieval of membrane components from vesicles released by exocytosis, etc).

There are thought to exist several types of endocytosis, one of the more important of which is clathrin-mediated endocytosis which is implicated, *inter alia*, in the cycling of neurotransmitters at synapses. Molecules destined to be internalised are concentrated in clathrin-coated pits which are then "pinched-off" to form vesicles. Steps leading to this include the synthesis of a curved clathrin lattice, providing a mechanical scaffold for vesicle formation, and the recruitment of a GTPase, dynamin, to the neck of the forming vesicle.

Small synaptic vesicles are specialised containers for the controlled release of neurotransmitters in response to nerve depolarisation. With the nerve terminals often far away from the cell body the neuron has become adapted to recycle these transmitter containers multiple times. The pathway for endocytosis is thought to occur predominantly by a clathrin-mediated mechanism [1]. This is supported both morphologically by clathrin coated vesicle profiles after nerve terminal stimulation and by the marked enrichment of the

molecular machinery for this pathway (including clathrin, adaptors and dynamin) in nerve terminals [2, 3].

The importance of endocytosis for the sustained activity of neurons is highlighted in *shibire*, a temperature sensitive mutant of dynamin in Drosophila [4, 5]. At the nonpermissive temperature there is an accumulation of endocytosis profiles and a rapid onset of paralysis. In this mutant the electron-dense collars around the necks of endocytosing vesicles resemble the dynamin rings found *in vitro* [6] or when lysed synaptosomes are treated with GTPγS [7]. Thus dynamin is likely to be a key protein in the "pinching off" stage of synaptic vesicle endocytosis.

As well as an N-terminal GTPase domain, dynamin contains a cluster of proline-rich sequences at its C-terminus that have the potential to interact with various Src homology 3 (SH3) domains. SH3 domains are modules commonly found in signal transduction and cytoskeletal proteins. They mediate protein-protein interactions by binding to proline rich sequences that adopt several turns of a type II polyproline helix. The polyproline region of dynamin (PRD) is important both in endocytosis and in its colocalisation with clathrin-coated pits, as has been demonstrated by transfection of deletion constructs into fibroblasts.

Amphiphysin, a major dynamin binding partner, is proposed to be involved in dynamin recruitment to the necks of coated vesicles [8-11]. Disruption of the interaction between these two proteins in either the lamprey giant reticulospinal synapse [10] or in fibroblasts [11] leads to an inhibition of clathrin-mediated endocytosis, probably through a blockade of dynamin recruitment. Amphiphysin is concentrated in nerve terminals and is present as a phosphorylated heterodimer of two isoforms (1 and 2) both of which bind to dynamin [12]. Synaptojanin and endophilin/SH3p4 have also been implicated in synaptic vesicle endocytosis by virtue of their concentration in nerve terminals and their interaction with components of the endocytosis machinery[13-15]. Synaptojanin additionally has a phosphatidyl inositol 5' phosphatase activity that may perform lipid modifications during endocytosis [13, 16].

In response to nerve terminal depolarisation dynamin is dephosphorylated by the calcium-calmodulin activated protein phosphatase calcineurin (also known as protein phosphatase 2B)

[17]. This has led to speculation that phosphorylation/dephosphorylation could regulate synaptic vesicle recycling [18]. In support of a role for calcineurin, this phosphatase is known to be enriched in nerve terminals where it co-localises with amphiphysin and indeed dephosphorylates amphiphysin and synaptojanin in parallel with dynamin [19]. On a wider scale, calcineurin activity in the brain is also implicated in synaptic plasticity, nerve regeneration and brain disease [20-22].

There is evidence to suggest that the interaction between dynamin 1 (hereafter referred to as dynamin) and amphiphysin plays an essential role in receptor-mediated endocytosis [11]. Dynamin binds to the SH3 domain of amphiphysin via the PSRPNR sequence in its polyproline domain.

Grabs *et al* [23] identified a peptide, (having the sequence PPPQVPSRPNRAPPG) from the dynamin polyproline domain, which was injected by Shupliakov *et al* (1997 Science 276, 259-263) into neurons who found that this caused an inhibition of synaptic vesicle endocytosis. However, this peptide was also found to inhibit Grb2 binding to dynamin, the function of which is not well understood. In addition, the peptide disclosed by Grabs *et al*, did not comprise a hydrophobic moiety.

Summary of the Invention

In a first aspect the invention provides a membrane permeable molecule which inhibits dynamin-mediated endocytosis in a eukaryotic cell, the molecule comprising a membrane permeable hydrophobic moiety, and a peptide moiety comprising the amino acid sequence QVPSRPNRAP. The sequence QVPSRPNRAP represents a portion of the prior art peptide, and specifically inhibits amphiphysin binding to dynamin.

The amino acid sequence may comprise trivial modifications such as amino acid residue substitutions, preferably of a conservative nature (e.g. substituting threonine for serine, or glutamine for asparagine). Preferably there are no more than 2 substitutions, more preferably no more than one substitution, and most preferably no substitutions.

The peptide moiety may also comprise one or more additional amino acid residues at the N-

and/or C-terminal. If such additional residues are present, their number is preferably small (e.g. no more than 5 additional residues at either terminal). For example, between 1 and 5 additional amino acid residues may be incorporated at either or both terminals.

If desired, the peptide moiety may comprise one or more D-amino acids - these are thought not to interfere with the endocytosis-inhibiting activity of the molecule, but render it more resistant to proteases and peptidases. In preferred embodiments, the molecule of the invention inhibits dynamin interactions with amphiphysin but does not affect interactions between dynamin and Grb2.

In some embodiments the molecule may comprise one or more labelling moieties, such as a fluorophore and/or a chromophore, or biotin and the like.

The hydrophobic moiety may be joined to a side chain of one of the amino acid residues of the peptide moiety. Serine, arginine, asparagine and glutamine all comprise side chains with groups which may be conveniently derivatised. However, it is preferred for a hydrophobic moiety to be joined to either (or both) of the terminal residues, typically via the terminal -NH_2 or -COOH groups. It will be apparent that the molecule may comprise a plurality of hydrophobic moieties, joined to a single peptide moiety. In addition, the molecule may comprise a dimer, oligomer or polymer comprising a plurality of the peptide and/or hydrophobic moieties.

The hydrophobic moiety is conveniently covalently coupled to the peptide moiety. A range of hydrophobic moieties are suitable for inclusion in the molecule of the present invention and include substituted or unsubstituted alkyl, alkenyl, alkoxy or aryl groups. Generally preferred are straight-chain (i.e. unbranched) substituted or unsubstituted alkyl, alkenyl or alkoxy groups, especially those comprising 10-24 carbon atoms, or more preferably 12-18 carbon atoms. In some instances, acetylation of the peptide moiety may be a convenient modification.

Desirably the molecule of the invention may be formed, at least in part, by a condensation reaction between a suitable hydrophobic fatty acid (e.g. lauric, myristic, palmitic, or stearic

acids) and a terminal amino acid of the peptide moiety. Conveniently the hydrophobic moiety is joined to the amino acid residue at the N terminal (as judged by reference to the sequence of dynamin protein).

In one embodiment, the molecule comprises the amino acid sequence QVPSRPNRAP, joined at the N terminal via a covalent bond to a myristoyl group. Other suitable hydrophobic groups are fatty acids which are present in membrane lipids of eukaryotic cells, and lipid moieties such as sphingosine.

The inventors have found that the presence of a negative charge on the residue at the C terminal (as judged by reference to the sequence of dynamin) inhibits its downregulatory effect on endocytosis. Accordingly, it is preferred to avoid the presence of a carboxyl group at the "C" terminal. This can be achieved in a number of ways, most conveniently by synthesis of the peptide moiety with an amino group in place of a carboxy group at the C terminal amino acid residue. It is therefore preferred that the peptide moiety has no negative charge at the "C" terminal at physiological pH (e.g. pH 6-8), and in some embodiments that there is no net negative charge on the peptide moiety (at physiological pH).

In some embodiments, the molecule may comprise a targeting moiety, such that the molecule is targeted to, and preferentially taken up by, particular cell types, such that endocytosis is preferentially inhibited in the target cell relative to non-target cells which may be present. Examples of targeting moieties include immunoglobulins or fragments thereof (e.g. Fv, Fab, scFv). Typically these targeting moieties would have a specific binding activity for a receptor molecule (such as a cell surface antigen, tumour-associated antigen or the like) expressed on the surface of a target cell. Alternative targeting moieties are hormones or other ligand molecules, or portions thereof, which retain specific binding activity for the cognate receptor expressed on the surface of a target cell.

The molecule of the invention is useful as an inhibitor of clathrin-mediated (specifically, dynamin-mediated) endocytosis. As such, the molecule has considerable usefulness as a research reagent for studying endocytosis, especially in *in vitro* culture systems (e.g. in cultured neurones or isolated synaptosomes).

Accordingly, in a second aspect, the invention provides a method of inhibting endocytosis in a eukaryotic cell *in vitro*, the method comprising the steps of : providing a culture of eukaryotic cells *in vitro*; and adding to the extracellular environment a molecule in accordance with the first aspect of the invention defined above. Generally, the eukaryotic cells will be mammalian cells, preferably simian, mouse, guinea pig, rat or human cells.

The method may further comprise the step of detecting and/or measuring the amount of endocytotic activity in the cells. Means of detecting and/or measuring endocytotic activity are known to those skilled in the art (e.g. as disclosed herein) - the most appropriate means may depend on the type of eukaryotic cells being investigated.

It will be apparent to those skilled in the art that a specific inhibitor of dynamin-mediated endocytosis may find a therapeutic use *in vivo*. Thus, in a third aspect, the invention provides a pharmaceutical composition for inhibiting endocytosis in a mammalian (preferably human) subject, the composition comprising a molecule in accordance with the first aspect defined above and a physiologically acceptable diluent, carrier or excipient (e.g. saline or phosphate-buffered saline solution). The composition will typically be substantially sterile, and may comprise additional components, such as liposomes.

An effective dose of the composition will be sufficient to provide between 0.1μg and 1000mg of the active molecule, typically between 10μg and 500mg. An optimum dose may be determined by routine trial and error by a person skilled in the art.

One potential use that could be envisaged is in neurological disorders where excitotoxicity is a problem, such as epilepsy, panic attacks etc. The molecule could lead to depression of neurotransmitter release: for example, the inventors have found that a molecule in accordance with the invention depresses synaptic transmission in rat brain slices, and may exert a similar effect in man. The molecule could also inhibit receptor sequestration, although the molecule would be unlikely to affect ionotrophic receptors since these are not down regulated by internalisation. However, the metabotrophic, G-protein coupled receptors, such as some of the glutamate receptors, are internalised and this class of receptor has been implicated in some disorders. Thus the molecule of the invention might be used to inhibit

internalisation/downregulation of G-protein coupled receptors and certain other cell surface receptors. In this regard, the inventors have already found that a molecule in accordance with the invention can inhibit transferrin uptake in COS cells and somatostatin receptor internalisation (data not shown).

The *in vivo* method of the invention may preferably be performed using a molecule in accordance with the first aspect which comprises a targeting moiety, as defined above. In this way, endocytosis may be inhibited preferentially in target cells in the subject's body.

In a fourth aspect the invention provides a method of inhibiting dynamin-mediated endocytosis in a mammalian subject, the method comprising the steps of: forming a pharmaceutical composition (preferably substantially sterile) comprising a molecule in accordance with the first aspect of the invention; and administering the composition to the subject.

The composition may be administered by injection (e.g. intravenous or intramuscular) or transdermally (e.g. by use of patches, dressings and the like). Alternatively the composition may be administered orally (e.g. in the form of a tablet, capsule or the like). In this instance it may be preferred to provide the composition in the form of an enteric-coated capsule for sustained gradual release of the active agent.

In a fifth aspect, the invention also provides a method of making a membrane permeable molecule which inhibits dynamin-mediated endocytosis, the method comprising the steps of: forming a peptide moiety comprising the amino acid sequence QVPSRPNRAP; and covalently joining a hydrophobic moiety to the peptide moiety.

The invention will now be further described by way of illustrative example and with reference to the accompanying drawings, in which:

Figure 1 shows a schematic representation of the structure of amphiphysin, and illustrates the amino acid sequence of a number of peptides corresponding to fragments of dynamin;

Figure 2 shows photographs of a number of Coomassie-stained SDS PAGE gels;

Figure 3A shows confocal microscopy images of synaptosomes loaded with a fluorescent dye (panel 1) and with the synaptosomes having subsequently released the dye (panel 2);

Figure 3B is a schematic representation of the assay method used by the inventors (step 1, loading synaptosomes with 100μM FM2-10; step 2, stimulation; step 3, washing and re-polarisation; step 4, second stimulation);

and Figure 4 is a graph of ΔF (arbitary units) against time (seconds), showing the results of an assay as illustrated in Figure 3B.

Examples

Example 1 : Mapping amphiphysin binding site on dynamin

At least seven proteins containing SH3 domains bind to dynamin *in vitro* via different polyproline sequences on the C-terminal ~ 100-residue Polyproline Region Domain (Okamoto *et al*, 1997 J. Biol. Chem. 272, 11629-11635). Isoform 1 of amphiphysin (Amph1) interacts with the sequence PSRPNR at a site partly overlapping the sequence reported to bind to the Grb2 N-terminal SH3 domain (Grabs *et al*, 1997; Okamoto *et al*, 1997; cited above). In order to identify the binding site for Amph2 and to investigate its relationship to that of Amph1 and Grb2, a number of peptides were designed and synthesised for use in binding studies with recombinant SH3 domains of Amph1, Amph2 and Grb2.

Accordingly, the DNA fragment corresponding to the SH3 domain of Amph2 (corresponding to amino acids 494-588 of Amph2 deposited at Genbank, Y13380) was amplified by PCR and cloned into the expression vector pET15b (Novagen) and expressed from the *E. coli* strain BL21 (DE3). For present purposes, residue 494 of the full length Amph2 was defined as residue 1 of the Amph2 SH3 domain. The resulting protein contained an N-terminal His6 tag and thrombin cleavage site, had an apparent molecular weight on SDS PAGE of approximately 14kDa. This fragment was also cloned into a pCMV-MYC eukaryotic

expression vector and into pGex4T for expression as an N-terminal GST fusion protein. The sequence encoding residues 595-683 of the SH3 domain of Amph1 was cloned and amplified by PCR in essentially the same way using the same vectors.

Rat Grb2 N-terminal SH3 domain was obtained by PCR from a rat brain cDNA library using primers as described previously (Gout *et al*, 1993 Cell 75, 25-36) and cloned into pGex4T and pCMV-MYC.

For large scale bacterial expression His6Amph2 SH3 single transformants were grown in 2TY medium supplemented with 120μg/ml ampicillin at 37°C. Expression was induced by the addition of IPTG to a final concentration of 0.3mM. Cells were harvested after 5 hours growth by centrifugation, resuspended in 30ml buffer per litre of culture (50mM Tris pH 7.5, 100mM salt, 10mM βmercaptoethanol 0.1mM PMSF 0.1mM benzamidine) and lysed by 2 passes through a French pressure cell. Protein was purified at 4°C by NiAgarose affinity, ion exchange (fast-flow Q-sepharose) and gel filtration (Superdex S200) chromatography giving a final yield of 20mg purified protein per litre of culture.

Figure 1 is a schematic representation of the common domain structure of Amph1 and Amph2, along with the sequence of the polyproline region of dynamin with which both isoforms interact. Sequences P1, P2, P4, P5 and P6 indicate the five peptides used for mapping the binding regions, and the solid bars indicate Grb2 N-terminal and amphiphysin SH3 domain binding sites. The ability of each of peptides P1-P6 to inhibit the interaction of dynamin with the SH3 domains of Amph1, Amph2 or Grb2 was tested *in vitro*, as described below.

All SH3 domains for use in protein-protein interaction assays were expressed in bacteria as N-terminal fusion proteins with glutathione-S-transferase (GST). Protein was purified from bacterial lysates by incubation with glutathione agarose beads in purification buffer (20mM HEPES pH7, 150mM NaCl 4mM DTT, 0.1mM PMSF 0.1mM benzamidine 5μg/ml E64) for 1hr at 4°C followed by extensive washing with purification buffer. 20μg of the purified fusion protein coupled to glutathione beads was then incubated with 0.5ml of rat brain extract under the desired conditions (presence or absence of peptide, pH) at 4°C. After washing

beads three times in purification buffer containing 0.1% TX-100, bound proteins were analysed by coomassie blue staining of 12.5% SDS polyacrylamide gels. (Rat brain extract was prepared by homogenising one rat brain in 15ml purification buffer in a glass-Teflon homogeniser followed by addition of Triton X-100 to a final concentration of 0.1% to complete lysis. Insoluble material was pelleted at 350,000g for 10 minutes.)

The results of the SDS PAGE analysis are shown in Figure 2. In Figure 2, the top panel shows results obtained when recombinant GST-SH3 domain fusion proteins were incubated with rat brain extract and GST agarose beads in the absence of peptide. The panels below show results obtained in the presence of 200μM concentrations of peptides P1, P2, P4, P5 and P6 respectively.

The only peptides that abolish amphiphysin binding were P2 and P4, which share the residues QVPSRPNR. It had previously suggested that the Amph2 binding site on dynamin might show different peptide-binding characteristics from that of Amph1 (Wigge *et al*, 1997 Mol. Cell. Biol. **8**, 2003-2015) but the new data demonstrate that the two isoforms do in fact bind to the same proline-rich motif on dynamin. Amphiphysin binding to this PSRPNR site is very specific, since peptides P1, P5 and P6 do not compete effectively for the amphiphysin-dynamin interaction. Clearly, the presence of two arginines on dynamin is crucial for the interaction of the SH3 domains of the amphiphysins. This contrasts with most other dynamin-binding SH3 domains; Grb2 binding is prevented only by P2.

In view of these results, peptide P4 was selected for further study.

Example 2: Synthesis of Myristoyl-Dynamin P4 (Myr P4).

A molecule in accordance with the invention was prepared as described below. The molecule consisted of a myristoyl group coupled to the N-terminal of a peptide moiety;

$H_3C(CH_2)_{12}CO$-GlnValProSerArgProAsnArgAlaPro-NH_2.

The Gln residue represents the N-terminal and Proline the C terminal of the peptide moiety,

by analogy with the natural sequence of dynamin. However, the inventors synthesised the peptide with an amino group on the C terminal proline residue, as this was expected to increase the stability of the molecule. Unexpectedly, it was also found that the presence of an amino group at the C terminal was desirable for optimum membrane permeability:- when the molecule was re-synthesised with a carboxyl group at the C terminal, the molecule was not taken up by cells. The inventors believe that the presence of a negative charge (the carboxyl group is negatively charged at physiological pHs i.e. about pH 6-8) at the C terminal is detrimental to uptake of the molecule by cells, so a carboxyl or other negatively-charged group at the C terminal is best avoided.

The synthesis was performed on a NovaSyn 'Crystal' peptide synthesizer using 100μmol of Fmoc-NovaSyn-KR resin, which automatically gives a C-terminal amide.

Side-chain protection was as follows: Arg(Pmc); Asn(Trt); Gln(Trt); Ser(tBu).

All residues except Arg, Ser and the C-terminal Pro were coupled for 1 hour using a 5-fold excess of Fmoc-OPfp ester in the presence of 1 equivalent of HOBt in DMF. Acylation reactions were monitored using UV absorbance monitoring at 304nmn.

The C-terminal proline was double-coupled for 2x2 hours using a 5-fold excess of Fmoc-OPfp ester in the presence of 1 equivalent of HOBt in DMF. Acylation reactions were monitored using UV absorbance monitoring at 304nmn.

Arg and Ser were coupled for 1 hour using a 5-fold excess of Fmoc-OBt ester generated *in situ* from the protected amino-acid, PyBOP, HOBt and Hünig's base in DMF. Acylation reactions were monitored using UV absorbance monitoring at 348nm.

Deprotection reactions were monitored using UV absorbance monitoring at 300nm is 20% Piperidine/DMF.

The N-terminus was myristoylated off-column for 2 hours using a 5-fold excess of Myristyl-OBt ester generated *in situ* from myristic acid, PyBOP, HOBt and Hünig's base in DMF.

The peptidyl-resin was cleaved using 94:3:3 (v/v/v) TFA/iPr3SiH/H20 for 2.5 hours and the crude product dissolved in a 1:1 mixture of 0.1%TFA in H_2O and CH_3CN containing 10% 0.1%TFA in H_2O.

If desired, the crude product can be purified by reverse-phase preparative hplc performed on a Vydac 208TP1022 C8 200x20mm column, as below.
Buffer A: 0.1% TFA/H20.
Buffer B: CH3CN containing 10% Buffer A.
The column was eluted isocratically with 40% Buffer B for 2 minutes then with a linear gradient of 40-90% over 25 minutes. Detection was at 215nm, flow rate 10ml/min. Pure fractions were combined and lyophilized.

The identity of the product was confirmed by reverse-phase analytical hplc, amino-acid analysis and matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry.

Example 3

The inventors wished to test the ability of the MyrP4 molecule to inhibit endocytosis *in vitro*. To do this, they investigated the effect of Myr P4 on synaptic vesicle recycling in rat brain synaptosomes, using a fluorescent dye, FM2-10, to assay endocytosis.

Synaptosome preparation

Rat brain synaptosomes were prepared essentially as described previously [24] but taking care of the following points. A rat brain was dissected on ice to remove the mid-brain and hind-brain, meninges and as much myelin as possible. This is particularly important as myelin in the synaptosome preparation traps large amounts of FM2-10 resulting in a high background fluorescence and thus a greatly decreased signal to noise ratio. The brain was then homogenised in approximately 20ml of 0.32M Sucrose, 20mM HEPES pH7.3 using 6 strokes with a Potter homogeniser at around 800rpm.

The brain homogenate was then centrifuged in a Sorvall SS-34 rotor for 2min at 5000rpm and the pellet discarded. The supernatant was centrifuged again in the same rotor for a

further 12min at 11,500rpm. The pellet was resuspended in approximately 8ml of 0.32M sucrose, 20mM HEPES and this was loaded onto percoll gradients (4%, 10%, 23%) and centrifuged at 18,000rpm for 11min in a Beckman SW40Ti rotor. The synaptosomes were removed from the interface between the 10% and 23% percoll, leaving any remaining myelin behind in the 4% percoll layer. The synaptosomes were diluted into HEPES buffered medium (HBM - 140mM NaCl, 5mM KCl, 5mM $NaHCO_3$, 1.2mM Na_2HPO_4, 1mM $MgCl_2$, 20mM HEPES, 10mM Glucose, pH7.3), centrifuged for a final time for 14min at 11,500rpm in the Sorvall SS-34 rotor and the pellet resuspended in approximately 4ml of HBM.

FM2-10 was used to follow endocytosis in isolated nerve terminals

FM dyes are highly fluorescent when inserted into lipid membranes but their fluorescence is negligible in aqueous solution (for review see [25]). When synaptosomes are incubated with FM2-10, the dye partitions into the membrane. A stimulus that causes synaptic vesicle endocytosis results in dye-loaded vesicles being internalised. Subsequent exocytosis releases internalised dye and a decrease in fluorescence is observed. Figure 3A shows confocal microscopy images of a synaptosome preparation that has been loaded with FM2-10 on stimulation of vesicle cycling. Active synaptosomes fluoresce due to the labelled vesicles they contain and can be clearly visualised after washing away excess dye (Panel 1). Exocytosis caused by a second depolarising stimulus results in the recycled synaptic vesicles releasing the trapped dye (Panel 2) and consequently the fluorescence is largely lost as the dye rapidly dilutes into the aqueous phase.

The field shown in Figure 3A is a selected one that demonstrates the phenomenon of destaining most effectively. In practice it was hard to obtain such images consistently and reproducibly. Synaptosomes adhere poorly to cover slips, additionally, due to their small size, it was usually difficult to find the same plane of focus on the same synaptosomes before and after any particular treatment. Also varying degrees of myelin contamination presented a problem. For these reasons, quantitative measurements of fluorescence changes were made using a large population of synaptosomes in a spectrofluorimeter as shown schematically in Figure 3B. In essence, synaptosomes were incubated with FM2-10 at 37°C (Step 1) and then vesicle endocytosis was stimulated as described above (Step 2). The dye loaded synaptosomes were then washed to repolarise and remove external dye (Step 3). Vesicle

cycling was then measured by a further stimulation (Step 4) in which dye labelled vesicles were released. Steps 3 and 4 in Figure 3B correspond respectively to panels 1 and 2 of Figure 3A. The decrease in fluorescence ΔF on dye unloading is a measure of the amount of dye that was endocytosed during the first stimulation (loading). By changing the conditions under which dye was taken up and subsequently measuring ΔF the inventors were able to determine the effects of those conditions on synaptic vesicle recycling.

Example 4

Synaptic vesicle recycling assay in rat brain cortical synaptosomes

Synaptosomes (500μl, prepared as described above) were diluted to 1ml in HBM in a stirred cuvette and incubated with FM2-10(100μM) and Ca^{2+} (normally 1.3mM) for 3min at 37°C. Vesicle cycling was then stimulated with 30mM KCl for 45s. The sample was then washed with two short 10s spins, to remove externally bound dye, and resuspended in 1ml fresh HBM to allow repolarisation. The synaptosomes are gently stirred at 37°C for approximately 10min to further remove dye from the plasma membrane. Labelled vesicles are then released during a second round of vesicle cycling, stimulated with 30mM KCl, 1.3mM$[Ca^{2+}]_o$ (external Ca^{2+} concentration).

The inventors measured the decrease in fluorescence when the dye is exocytosed in a Fluoromax-2 spectrofluorimeter, exciting at 467nM and collecting at 550nM. The decrease in fluorescence (ΔF) on dye unloading is a measure of the amount of dye that was endocytosed during the first stimulation (loading). FM-143 has previously been used to measure exocytosis in synaptosomes [26] but FM2-10 is more easily washed from external membranes giving a lower background signal.

To test the effect of Myr P4, the compound was added to the synaptosome preparations, prior to initial stimulation, at a concentration of 50μM. As a control, a myristoylated scrambled peptide with the same amino acids, but in a different order (QPPASNPRVR) was also tested.

The results are shown in Figure 4, which is a graph of ΔF (arbitary units) against time (in seconds). It was found that 50μM Myr P4 inhibited recycling of FM2-10 by 85% $\pm$ 10%

(n = 3), whilst the scrambled peptide had no discernible effect.

Additional experiments measuring glutamate release from the synaptosomes (via an enzyme-coupled assay, performed as described previously [27] demonstrated that exocytosis of glutamate was not affected (data not shown) indicating that MyrP4 exerts an inhibitory effect on the endocytotic part of the synaptic vesicle cycle and does not inhibit exocytosis.

The results obtained by the inventors demonstrate that molecules in accordance with the invention can be delivered to the extracellular environment and pass through the cell membrane to exert an effect on an intracellular target (dynamin/amphiphysin interactions) and thereby inhibit dynamin-mediated endocytosis.

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CLAIMS

1. A membrane permeable molecule which inhibits dynamin-mediated endocytosis in a eukaryotic cell, the molecule comprising a membrane permeable hydrophobic moiety, and a peptide moiety comprising the amino acid sequence QVPSRPNRAP.

2. A molecule according to claim 1, comprising one or more additional amino acid residues at one or both ends of the peptide moiety.

3. A molecule according to claim 1 or 2, wherein the peptide moiety does not have a negative charge at physiological pH.

4. A molecule according to any one of claims 1, 2 or 3, wherein the peptide moiety does not have a negatively charged group at the C terminal, at physiological pH.

5. A molecule according to any one of the preceding claims, wherein the hydrophobic moiety comprises a substituted or unsubstituted alkyl, alkenyl, alkoxy or aryl group.

6. A molecule according to any one of the preceding claims, wherein the hydrophobic moiety comprises 10-24 carbon atoms.

7. A molecule according to any one of the preceding claims, wherein the hydrophobic moiety comprises 12-18 carbon atoms.

8. A molecule according to any one of the preceding claims, wherein the hydrophobic moiety is covalently coupled to the N terminal amino acid residue of the peptide moiety.

9. A molecule according to any one of the preceding claims, wherein the hydrophobic moiety comprises an unbranched hydrocarbon chain which may be substituted or unsubstituted.

10. A molecule according to any one of the preceding claims, additionally comprising a

labelling moiety and/or a targeting moiety.

11. A method of making a membrane permeable molecule which inhibits dynamin-mediated endocytosis, the method comprising the steps of: forming a peptide moiety comprising the amino acid sequence QVPSRPNRAP; and covalently joining a hydrophobic moietyto the peptide moiety.

12. A method according to claim 11, performance of which results in a molecule according to any one of claims 1-10.

13. A method according to claim 11, wherein a fatty acid is joined to the peptide moiety via a condensation reaction.

14. A method of inhibting endocytosis in a eukaryotic cell *in vitro*, the method comprising the steps of: providing a culture of eukaryotic cells *in vitro*; and adding to the extracellular environment a molecule in accordance with any one of the preceding claims.

15. A method according to claim 14, wherein the cells are mouse, rat or human cells.

16. A method according to claim 14 or 15, further comprising the step of detecting and/or measuring the amount of endocytotic activity in the cells.

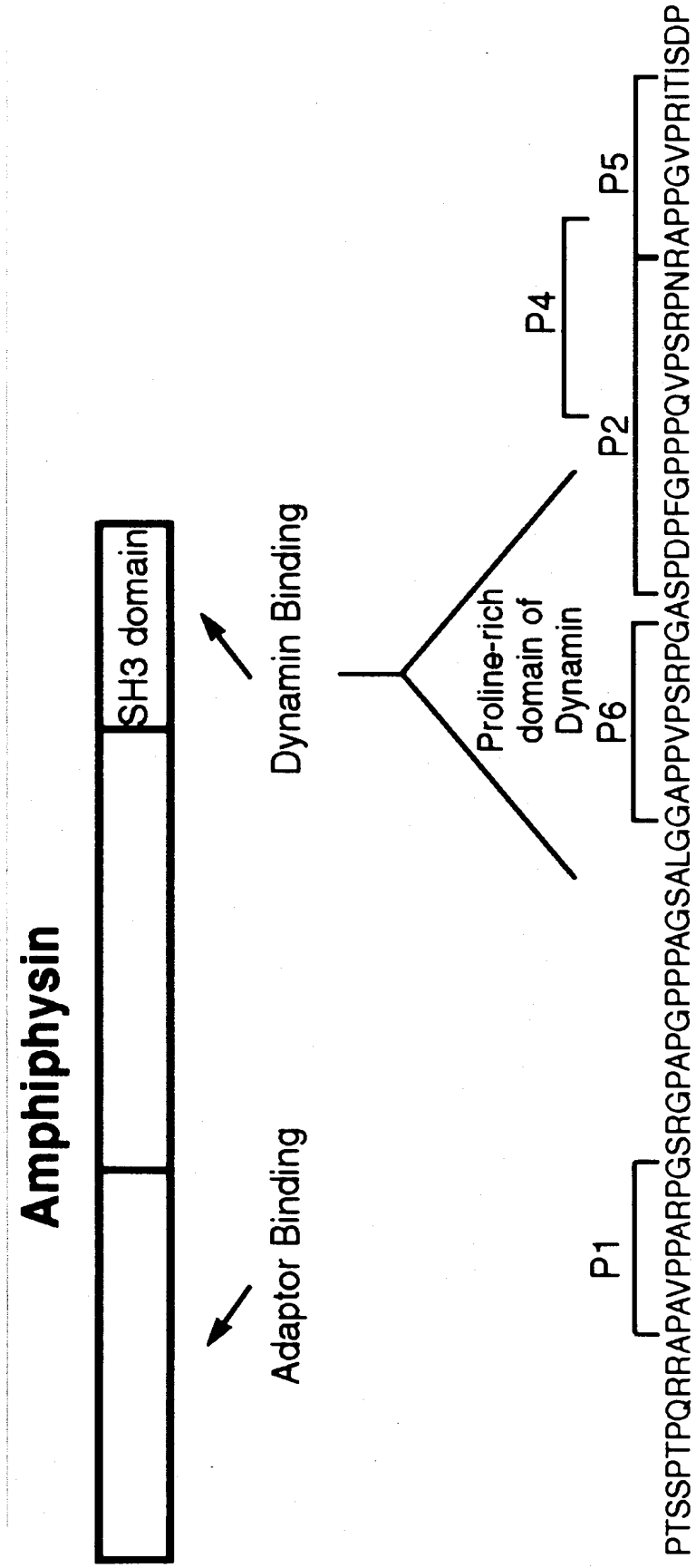
17. A pharmaceutical composition for inhibiting endocytosis in a mammalian subject, the composition comprising a molecule in accordance with any one of claims 1 to 10, and a physiologically acceptable diluent, carrier or excipient.

18. A composition according to claim 17, in the form of an injectable liquid, or a tablet.

19. A composition according to claim 17 or 18, comprising a liposome.

20. A method of inhibiting dynamin-mediated endocytosis in a mammalian subject, the method comprising the steps of: forming a substantially sterile composition comprising a

molecule in accordance with any one of claims 1 to 10; and administering the composition to the subject.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Dynamin Binding
Adaptor Binding
Proline-rich domain of Dynamin
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Amph
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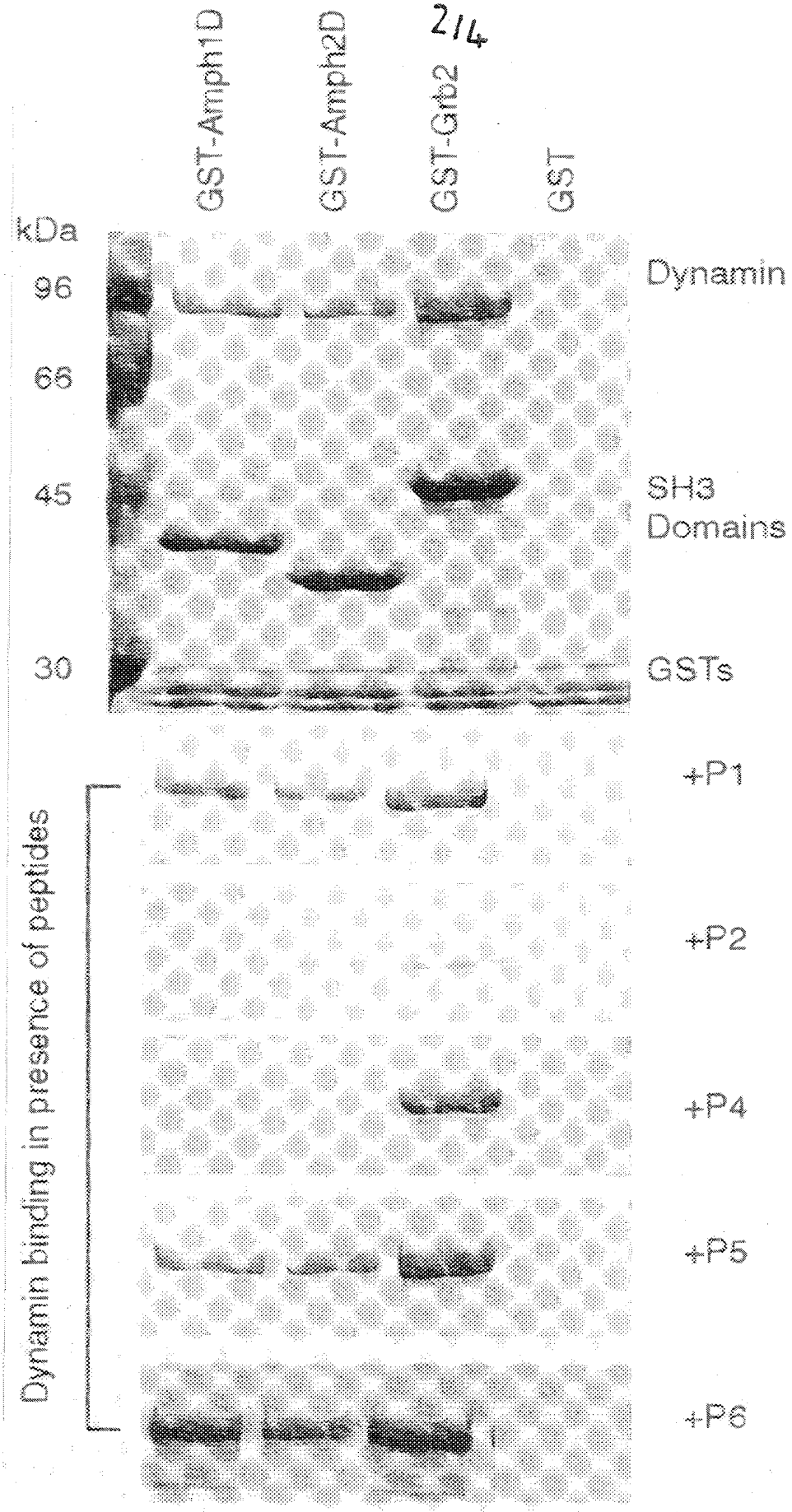
Fig. 1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kDa
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66
45
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Dynamin
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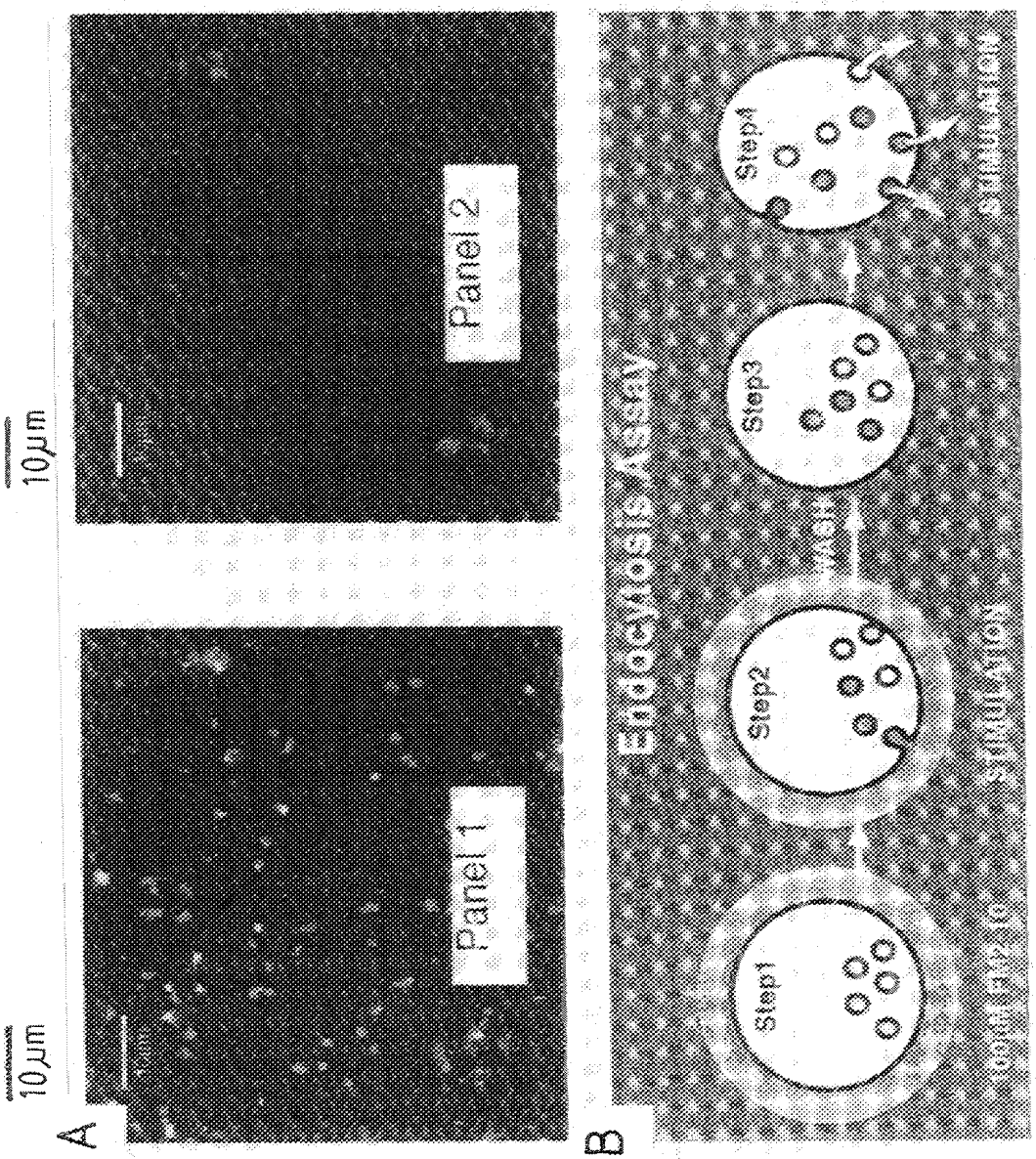
GSTs
+P1
+P2
+P4
+P5
+P6
Dynamin binding in presence of peptides

Fig. 2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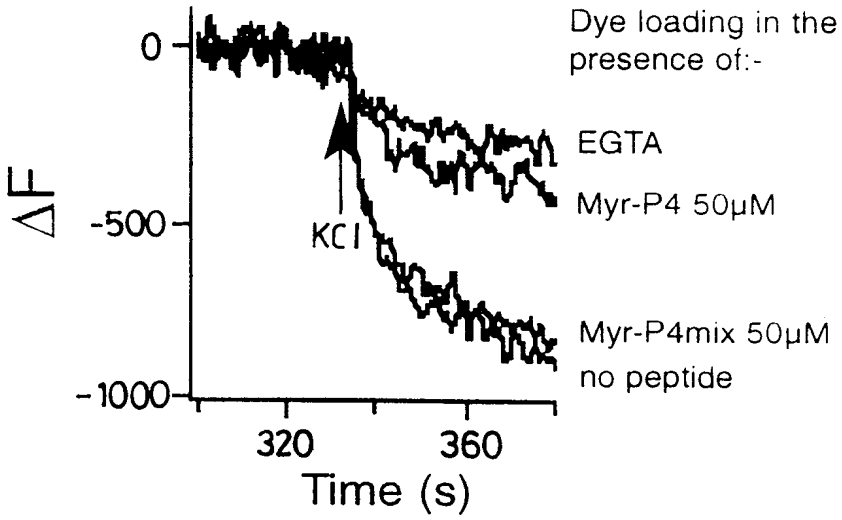
Panel 1
10μm
Panel 2
B
Endocytosis Assay
Step1
Step2
WASH
Step3
Step4

Fig. 3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-500
-1000
ΔF
KCl
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EGTA
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Myr-P4mix 50μM
no peptide

Fig. 4

SEQUENCE LISTING

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